# A Convenient Colorimetric Assay for α-Methylacyl-CoA Racemase (AMACR; P504S) And Testing Of Inhibitors

Maksims Yevglevskis,[a] Guat Lee,[a] Amit Nathubhai,[a] Tony D. James,[b] Michael D. Threadgill,[a] Timothy J. Woodman[a] and Matthew D. Lloyd[a,‡]

Medicinal Chemistry, Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, U.K. ‡email: M.D.Lloyd@bath.ac.uk
Department of Chemistry, University of Bath, Claverton Down, Bath, BA2 7AY, U.K.

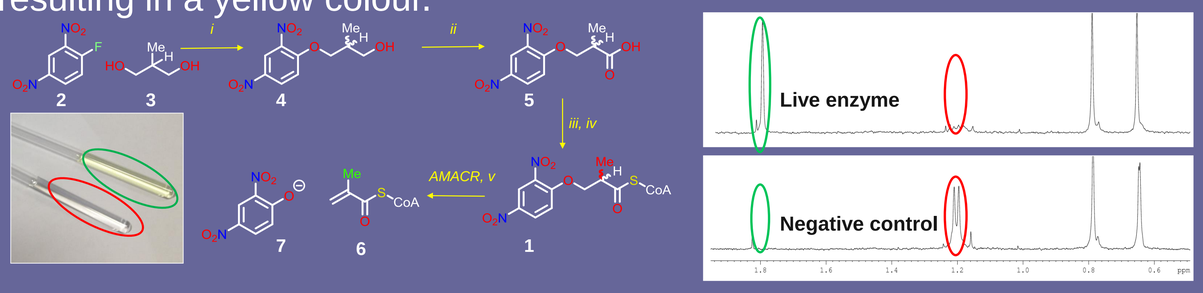
## Introduction

Branched-chain fatty acids are common in the diet and similar structures are found in medicines such as Ibuprofen and related drugs. Metabolism of branched-chain fatty acids requires that the centres bearing the methyl groups possess *S*-stereochemical configuration, but those with *R*-configuration are produced in the body and are found in the diet. Ibuprofen and related drugs require *S*-configuration for their anti-inflammatory properties, but these drugs are usually given as a mixture of *R*- and *S*-enantiomers. The enzyme α-methylacyl-CoA racemase (AMACR) catalyses *R*- to *S*- conversion of 2-methylacyl-CoA derivatives of fatty acids enabling β-oxidation. Similarly, acyl-CoA derivatives of Ibuprofen and similar drugs are converted, resulting in pharmacological activation.[1,2]

AMACR levels are increased in all prostate cancers, some colon cancers and other cancers.[1-3] In prostate cancer, higher AMACR levels result in higher proliferation rates[4] and androgen-independent growth[5] and AMACR is recognised as a novel drug target. However, few inhibitors have been identified, largely due to the difficulties in measuring enzyme activity which makes it difficult to quantify drug potency.[1] AMACR catalyses the irreversible elimination of hydrogen fluoride from 3-fluoro-2-methylacyl-CoA substrates,[6] but translating this reaction to a convenient colorimetric or fluorometric assay has proven difficult.[3] 4-Nitrophenol derivatives are commonly used as colorimetric substrates for enzymes. This study reports the synthesis of a 2,4-dinitrophenol-containing AMACR substrate and the characterisation of known AMACR inhibitors using a convenient colorimetric microtitre plate assay.

## Results and Discussion

2,4-Dinitrophenol is fully ionised at neutral pH giving a yellow colour and has a similar *p*Ka to HF, which is eliminated from known AMACR substrates. Therefore an acyl-CoA derivative **1** containing 2,4-dinitrophenol was investigated. Reaction of **2** with alcohol **3** to give **4** followed by oxidation gave the racemic acid **5,** which was converted to the desired substrate **1** (Scheme 1). Incubation of **1** with recombinant human AMACR 1A resulted in formation of unsaturated product **6** and 2,4-dinitrophenol **7** resulting in a yellow colour.




**Scheme 1:** Synthesis of novel substrate 1 and reaction with AMACR. *Reagents & conditions*: i. Na metal; ii. Jones oxidation; iii. CDI, DCM; iv. CoA-SH, $NaHCO_{3\ aq}$./THF (1:1); v. $NaH_2PO_4$-NaOH, pH 7.4, *ca*. 77% $^2H_2O$.

AMACR was active around neutral pH and retained full activity in the presence of 8% (v/v) DMSO. Kinetic analysis of substrate 1 showed that Michaelis-Menten kinetics were observed (Figure 1), with the following parameters: $K_m$ = 56 ±4.5 μM; $V_{max}$ = 112 ± 4 nmol.min.$^{-1}$mg$^{-1}$; $k_{cat}$ = 0.088 $s^{-1}$; $k_{cat}/K_m$ = 1571 $s^{-1}$ $M^{-1}$. This shows that substrate **1** is converted with ~44% of the efficiency of 3-fluoro-2-methyldecanoyl-CoA and was significantly more efficient than 'racemisation' of 2-methyldecanoyl-CoA (as judged by $k_{cat}/K_m$).[6]

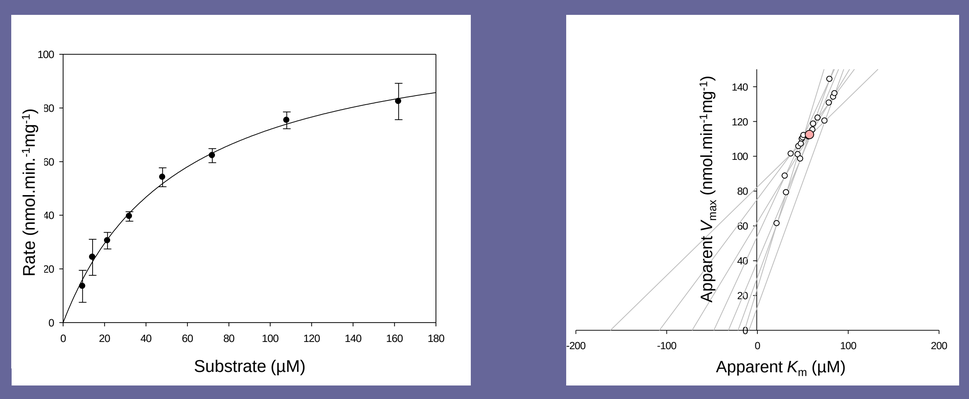



**Figure 1:** Kinetic analysis for substrate **1**.

The known inhibitor Rose Bengal[7] was tested to validate the method for characterisation of inhibitors (Figure 2). A dose-response curve was efficiently produced using a microtitre plate assay.

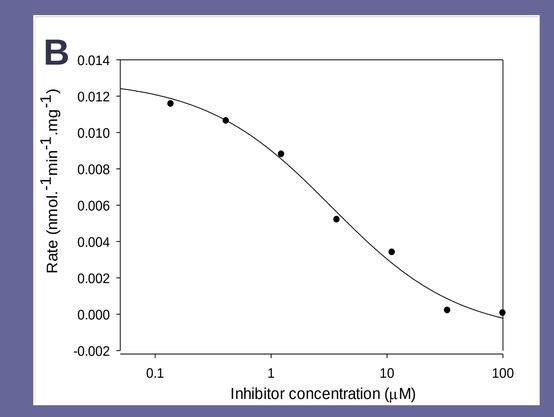



**Figure 2:** AMACR inhibition assay using Rose Bengal as an inhibitor. A. 96-Well plate showing colour change; B. Dose-response curve for Rose Bengal.

A number of other known AMACR inhibitors and substrates were tested using a dose-response curve at a fixed substrate concentration of 40 μM. Ibuprofenoyl-CoA and related compounds are known substrates and should behave as competitive inhibitors. All of these compounds inhibited the enzyme with $IC_{50}$ values of *ca.* 300-500 nM. 2-Methyldecanoyl-CoA also inhibited the reaction, and was *ca.* 4x more potent than decanoyl-CoA. Inhibition was decreased in acyl-CoA esters with shorter alkyl chains. The best acyl-CoA inhibitor was *N*-dodecyl-*N*-methylcarbamoyl-CoA,[8] which was ~500 – 1000 x more potent than the other acyl-CoA inhibitors (as judged by $IC_{50}$ values). The non-specific protein modifying reagents reported by Wilson *et al*.[7] also inhibited the enzyme; in contrast to previous reports Ebselen behaved as a time- and concentration-dependent inactivator with a rate constant of 114 $M^{-1}$ $s^{-1}$.

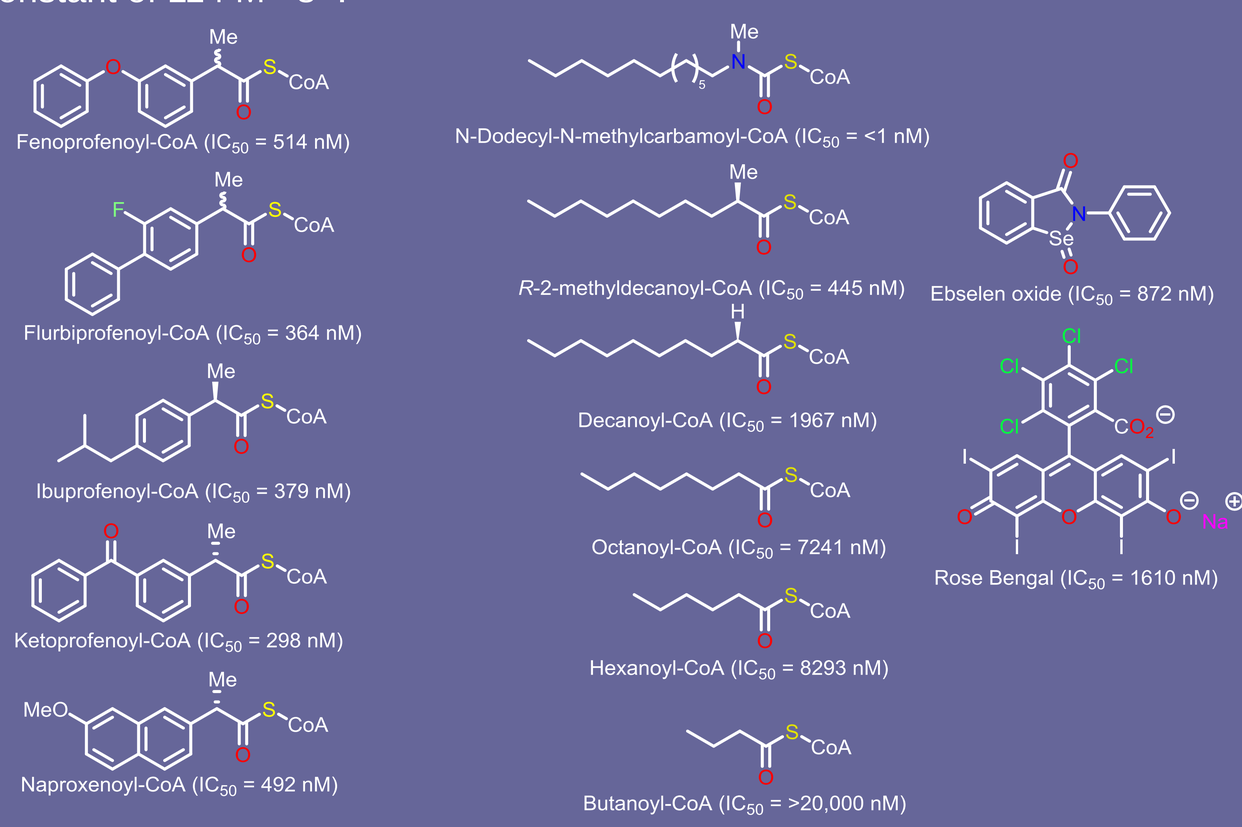



**Figure 3:** Selected acyl-CoAs and protein modifying agents shown to inhibit the conversion of substrate **1** to **6** and **7** by AMACR using the colorimetric assay.

## Conclusions

The colorimetric substrate **1** provides a convenient method for assaying AMACR and determining the behaviour and potency of inhibitors. AMACR is a promising drug target for prostate and other cancers, but until now it has been under-exploited because of the difficulties in determining enzyme activities. Inhibitors previously reported in the literature are largely limited to rationally designed acyl-CoA esters, which do not comply with Lipinski guidelines.[9] This new assay will facilitate the testing and development of drugs by structure-based design, rational design and lends itself to screening approaches. The latter should allow identification of inhibitors with good drug-like properties.

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